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Genome-wide profiling of the expression of serum derived exosomal circRNAs in patients with hepatic alveolar echinococcosis

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ABSTRACT

The patients with hepatic alveolar echinococcosis is poorly detected due to invasive and slow growth. Thus, early diagnosis of hepatic alveolar echinococcosis is so important for patients. Circular RNAs are crucial types of the non-coding RNA. Recent studies have provided serum-derived exosomal circRNAs as potential biomarkers for detection of various diseases. The clinical importance of exosomal circRNAs in hepatic alveolar echinococcosis have never been explored before. Here, we investigated the serum-derived exosomal circRNAs in the diagnosis of hepatic alveolar echinococcosis. Firstly, High-throughput Sequencing was performed using 9 hepatic alveolar echinococcosis and 9 control samples to detect hepatic alveolar echinococcosis related circRNAs. Afterwards, bioinformatic analyzes were performed to identify differentially expressed circRNAs and pathway analyzes were performed. Finally, validation of the determined circRNAs was performed using RT-PCR. The sequencing data indicated that 59 differentially expressed circRNAs; 31 up-regulated and 28 down-regulated circRNA in hepatic alveolar echinococcosis patients. The top 5 up-regulated and down-regulated circRNAs were selected for validation by RT-qPCR assay. As a result of the verification, circRNAs that were significantly up- and down-regulated showed an expression profile consistent with the results obtained. Importantly, our findings suggested that identified exosomal circRNAs could be a potential biomarker for the detection of hepatic alveolar echinococcosis serum and may help to understand the pathogenesis of hepatic alveolar echinococcosis.

1. Introduction

Hepatic echinococcosis (HE) caused by *Echinococcus* spp. transmitted from carnivores is known as a severe zoonotic disease that formed into hepatic cystic echinococcosis (HCE) and hepatic alveolar echinococcosis (HAE). It continues to be a serious public health issue around the world. HAE is uncommon infection which is characterized by poor prognosis but it is the most life-threatening compare to HCE (Nunnari et al., 2012; Ya-Min et al., 2018). The disease may detected with some imaging techniques such as ultrasound and serological parameters. There are four treatment options. Therse are surgery, PAIR (Puncture, aspiration, injection, re-aspiration), drug treatment, and observation. It is reported that HAE surgery is to be long operation time, high operation difficulty, several postoperative complications and slow postoperative recovery. The nutritional status of HAE patients is also important for the success operation (Brady et al., 2003).

The disease is progressed by a slow same as hepatocellular carcinoma. In addition, HAE could be determined as “worm cancer” and “parasitic liver cancer”(Parsak et al., 2007). Approximately 98% of cases, HAE leads to destructive hepatic lesions and is characterized by unlimited grown. The liver can not carry out synthesis and metabolism of some nutrients such as albumin due to damaged by HAE. Low blood albumin levels lead to loss of body weight, a poor general condition of patients and the inability to tolerate surgical treatment (Salm et al.,

Abbreviations: HE, Hepatic echinococcosis; HCE, hepatic cystic echinococcosis; HAE, hepatic alveolar echinococcosis; circRNAs, Circular RNAs.

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2019). Many cases of HAE are recognized at forward stages becuase clinical symptoms may remain silent in about 10 years of incubation period, Several pathological cases could be observed including septicemia, abscess, recurrent cholangitis, and portal hypertensive gastrointestinal bleeding in untreated people (Aliakbarian et al., 2018). It is provided that correlated factors includes CD44, Soluble ST2 (sST2), Plasma IL-23, IL-27, and IL-5 for metastasis and prognosis for patients with HAE were identified (Wang et al., 2015; Tuxun et al., 2018; An et al., 2020; Apaer et al., 2021). However, data on metastatic and pronostic marker are still so poor.

Recently, various studies have performed investigation on understanding the function of exosomes in cellular communication, development, differentiation, cell signaling, viral replication, tumor progress, tumor microenvironment, immune system (Zhang and Wang, 2015; Yu et al., 2018). Exosomes could be used in practical applications and clinical diagnososis (Alenquer and Amorim, 2015). Briefly, exosomes are considered to be a source of novel biomarkers due to the specific cargo loaded by their progenitor cells (Wang et al., 2019).

The recent advances in sequence techniques provide researchers to investigate the complex network of coding and noncoding transcriptome. Circular RNAs (circRNAs) are a class of endogenous noncoding RNAs. CircRNAs are usually produced by many genes and have a 5′ cap and 3′ tail structure unlike other noncoding RNAs and are characterized by a covalently closed loop structure formed by back-splicing event. CircRNAs modulate the gene expression interacting with RNA binding proteins (RBPs) (Ashwal-Fluss et al., 2014; Conn et al., 2015; Starke et al., 2015). Importantly, It has been reported that circular RNAs play a role in the onset and progression of many diseases such as some kinds of cancers, cardiovascular diseases, and neurological diseases, undergo expression changes in different stages of diseases, and could be molecular biomarkers (Floris et al., 2017; Li et al., 2018b; Zhong et al., 2018).

Splicing is crucial process for mRNA maturation and translation mechanism. It may generate various RNA types or isoforms which could play important roles in several biological functions (Braunschweig et al., 2013). Several human genes (over 95%) are alternatively spliced and different isoforms of which can be generated. Both splicing and alternative splicing mechanism are regulated by *trans*-regulatory factors and *cis*-regulatory elements (Wang et al., 2008). CircRNAs are occured mediated by unique type of splicing as called back-splicing which the 5′ terminus of a pre-mRNA upstream exon is non-colinearly spliced with the 3′ terminus of a downstream exon (Enuka et al., 2016). Most important function of circRNAs is inhibition of miRNA activity which post-transcriptionally regulates mRNA expression. Another function of circRNA is protein decoys to effect the cellular function. Thus the relation between the molecular/signaling mechanism of mRNA expression and circRNA could be consider to reveal unknown biological mechanism, pathogenesis of diseases, and complex mechanism of immun defense. CircRNAs participate in cell proliferation, epithelial-mesenchymal transition (EMT), cancer progression, pluripotency, early lineage differentiation, and SRY sex determining (Yu and Kuo, 2019).

Recent studies have revealed that circRNAs are enriched and stable in exosomes. Exosomal circRNAs may be localized in platelet-derived extracellular vesicles, hepatic cells, and pancreatic cancer cells (Dai et al., 2018; Li et al., 2018a; Preußer et al., 2018). Studies have suggested that it is possible that cells may transfer circRNAs by excreting them in exosomes (Choi and Lee, 2016; Lasda and Parker, 2016). Whereas there are a lot of studies on cancer and other human diseases related exosomal circRNA, no studies on hepatic alveolar echinococ-related exosomal circRNAs in humans. Current study provides that exosomal circular RNAs from healthy humans and humans with hepatic alveolar echinococcal disease were detected and characterized using the RNA sequencing.

2. Material and methods

2.1. Clinical samples

Present study was performed to investigate HAE related exosomal circRNAs as potential biomarkers. 18 serum samples were collected from HAE patients; 9 samples for sequencing and 9 samples for confirmation. 18 serum samples were also collected from healthy people who there was no medical history of HAE and other diseases; 9 samples for sequencing and 9 samples for conformation. We used three biological replicas for each experiment including RNA-seq and RT-PCR. All samples were collected from Atatürk University, Faculty of Medicine between 2019 and 2020. Patients with HAE were detected by imaging systems (especially ultrason), and samples were collected prior to any surgery and therapies. Information sheet was obtained from patients and controls before serum samples were collected. The study protocol was approved by the Clinical Research Ethics Committee of Atatürk University Hospital.

2.2. Serum samples

5 mL of blood samples were picked up from each participant was centrifuged at 3,000 rpm for 10 min at 4 °C (Xie et al., 2020), within the sterile eppendorf tubes. Later on the serum was filtered with 0.22 μm (Millex-GP Syringe Filter Unit) and was stored at − 80 °C for the future experiment.

2.3. Exosome extraction

We used exoEasy Maxi Kit (Qiagen, Germany) to extract the exososmes from serum samples. All stesp were performed according to the manufacturer's instructions. Briefly, serum samples were filtered to exclude particles larger than 0.8 μm Millipore Millex-AA. 500 μL buffer XBP was added to serum samples and the sample/XBP mix warm up to room temperature. 1 mL of the sample/XBP mix onto the exoEasy spin column and centrifuge at 1000 × g for 2 min. 1 mL buffer XWP and centrifuge at 5000 × g for 5 min to remove residual buffer from the column. The spin column was transfered to a fresh collection tube. 50 μL Buffer was added to the membrane and incubate for 5 min and then centrifuge at 500 × g for 5 min to collect the eluate. the exoEasy spin column membrane was Re-applied the eluate and incubate for 5 min. Lastly, it was centrifuged at 5000 × g for 5 min to collect the eluate and transfer to 1.5 mL eppendorf tube. Isolated exosomes were confirmed with Western Blot analysis (CD9 and CD63) (Mathieu et al., 2021) (Figure S1). After verification, exosomes was stored at − 80 °C for future experiment.

2.4. High-Throughput sequencing

9 samples from HAE patients were seperated into 3 groups and also 9 serum samples of healthy controls were also divided into 3 groups for sequencing. Thus, three sets of serum samples from HAE patients and three healthy control samples were obtained for sequencing. RNA-seq were carried out by Novogene (UK) Company Limited, United Kingdom. Briefly, the Ribo-Zero rRNA Removal Kit (Illumina, San Diego, CA, USA) and the CircRNA Enrichment Kit (Cloud-seq, USA) were used to elemiante the rRNA and get more circRNAs. RNA libraries were prepared with TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA, USA), and the quality and quantity of the libraries were analysed with the BioAnalyzer 2100 system (Agilent Technologies, Inc., Santa Clara, CA, USA). After cDNA were produced, the cDNA products were sequenced with Illumina HiSeq™ 4000 Sequencer (Illumina, San Diego, CA, USA) following the manufacturer's instructions.

2.5. Bioinformatic analysis of CircRNAs

All reads were acquired from Illumina HiSeq 4000 sequencer, and the quality control of reads were addressed by Q20 and Q30. The 3′adaptor was trimmed with Cutadapt software (v1.9.3), also the poor-quality reads was removed with Cutadapt software (v1.9.3). The high-quality reads were aligned to the reference genome/transcriptome by using STAR software, and CIRCexplorer2 software was used to identified circRNAs. The identified circRNAs were annotated with Circ2Trait database and circBase database. The differentially expressed circRNAs were detected with Edger software (DESeq2 R package). The functions of differentially expressed circRNAs were predicted by using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (KOBAS software).

2.6. Prediction of CircRNA-miRNA-mRNA network

Targetscan and Miranda were used to predict the miRNA binding sites and target mRNAs. For circRNAs, we observed the estimated miRNAs and target genes for each miRNA. CircRNA-miRNA-mRNA network was built with Cytoscape software to identify the interactions between these molecules.

2.7. RT-PCR

Total exosomal RNA was isolated with the miRNeasy Serum/Serum Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total RNA concentration was measured by NanoDrop spectrophotometer (Epoch Microplate Spectrophotometer) at 260 nm. Then, cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen, Germany) following the the manufacturer's instructions. The relative gene expression of RNA was determined using the Rotor-Gene Q MDx 5plex HRM (CA). β-actin was used a internal control (Li et al., 2019a; Li et al., 2019b). The relative expressions levels of circRNAs were measured by using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primer sequences were shown in Table S1.

2.8. Statistical analysis

GRAPHPAD PRISM 8 (San Diego, CA, USA) was used to create Scatter diagrams. We used SPSS statistics program (IBM, Chicago, IL, USA) for RT-PCR analysis. p less than 0.05 was considered to be

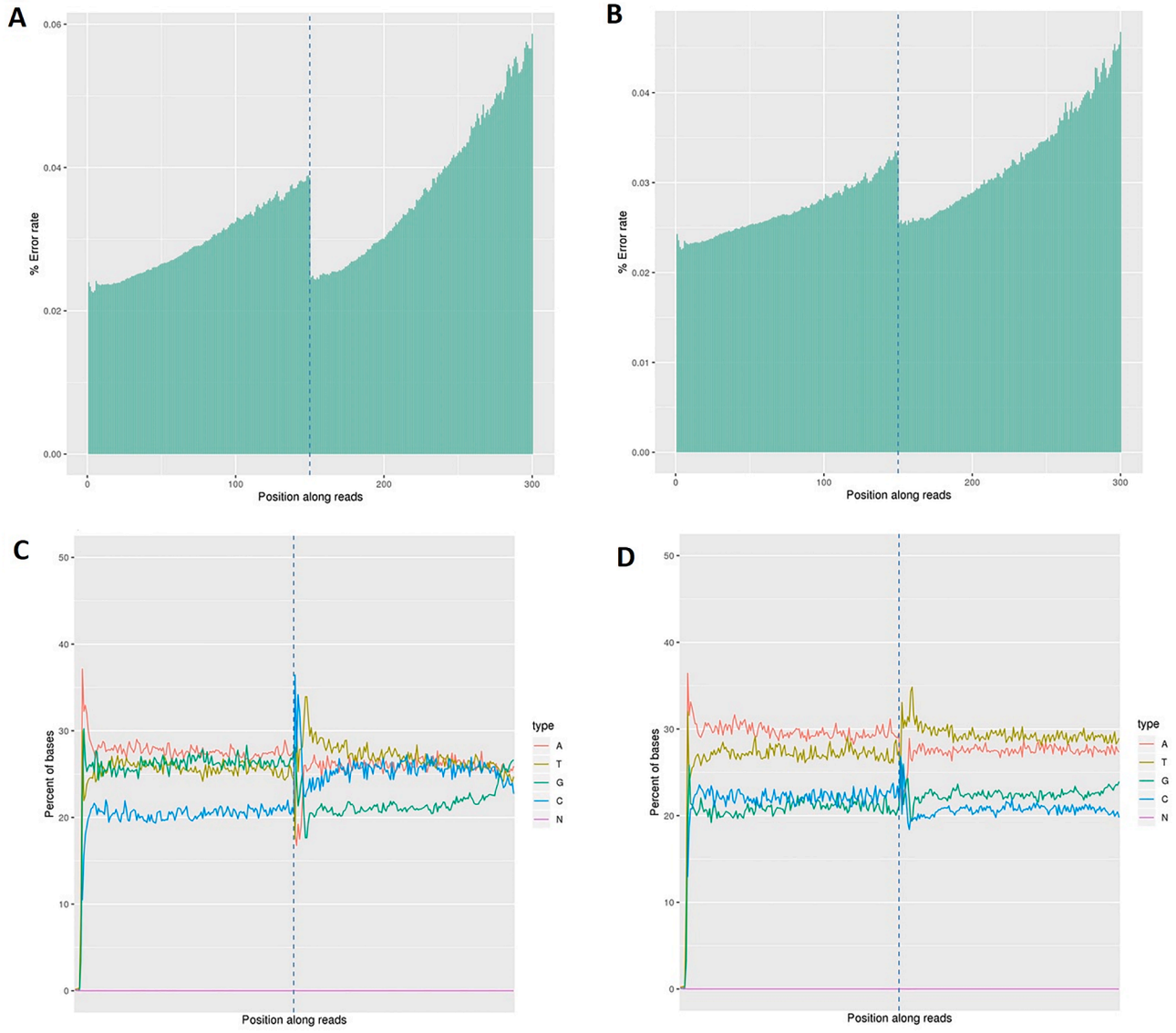

Fig. 1. Distribution of sequencing error rate and GC-content. The horizontal axis represents the position of reads, and the vertical axis represents the error rate. Horizontal axis for reads position, vertical axis for single base percentage. Different color for different base type. A) Control, B) Patient, C) Control, D) Patient.

statistically significant.

2.9. Data availability

The datasets from current study were submitted to NCBI (https://www.ncbi.nlm.nih.gov). Relevant data can be accessed from this site. (GSE183607).

3. Results

3.1. Quality control of sequencing results

RNA sequencing (RNA-seq) was performed to detect exosomal circRNAs in serum samples from HAE patients and healthy humans. One of the most important criteria in sequencing is that the sequencing results meet the desired criteria. The error rates and QC rates of the sequencing results performed in this study were revealed. Error rates were found to be approximately 0.03%, and QC rates were approximately 45% (Fig. 1A-1D). In addition, the Q20 ratio of the sequencing results was approximately 96%, and the Q30 ratio was approximately 90% (Table S2).

3.2. CircRNAs expression profiles

In total, 2203 circRNAs were detected. The detected RNAs were 37.2% intronic, 33.4% exonic, 14.7% antisense, 4.2% intergenic, and 10.5% sense overlapping (Fig. 2A, Figure S2). When the chromosome distributions of circRNAs were examined, it was observed that they were generally localized in all chromosomes. CircRNAs were found to be particularly concentrated on the 1–14 and X chromosomes. In addition, it was found that the Y chromosome contains 8 circRNAs (Fig. 2B-2C). The lengths of the identified circRNAs were found to be less than 2000 nt and most of them less than 500 nt (Fig. 3A-3F). The FPM distribution and intensities of these identified circRNAs are shown in Fig. 4.

3.3. Differential expression profiles of CircRNAs

A statistical important fold change was ≥ 2.0 and P-value ≤ 0.05. We detected that 59 circular RNAs were significantly different expressed. A heatmap (Fig. 5B) and a volcano plot (Fig. 5A) of all the differentially expressed circRNAs were constructed to show circRNA expression profiles. 802 circRNAs were expressed in Patiens, 798 circRNAs were expressed in the controls, 603 circRNAs were co-expressed in both patients and controls (Fig. 5C). Compared ven diagram analysis is shown in Figure S3.

Among the 2203 identified circRNAs, 31 were significantly up-regulated and 28 were significantly down-regulated (Fig. 5A) (Table S3). High up-regulated circRNAs hsa_circ_0002204, hsa_circ_0002290, hsa_circ_0001854, hsa_circ_0002436 and hsa_000-circ_2249 , down-regulated circRNAs hsa_circ_000832, hsa_circ_000160, hsa_circ_0001332, hsa_circ_000524, and hsa_circ_0003988 (Table S4, S5, and S6). These circRNAs were confirmed by RT-PCR. The comparation of sequence data and RT-PCR results were performed. We found that the results were consistent (Figure S4).

3.4. Detection of potential functions

Current studies suggest that circRNAs can modulate the expression of the several genes (19, 20). We realized Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis for circRNAs to identify their potential functions. We evaluated the significantly up-regulated and down-regulated circRNAs on GO analysis. Two significantly enriched GO terms in biological processes detected were protien containing complex assembly and biosynthetic process. It was shown that four most significantly enriched GO terms were cytosol, nucleus, organelle, and nucleoplasm. Three significantly enriched GO terms were enzyme binding, ligase activity, and kinase activity (Fig. 6A and B). The KEGG analysis were used to predicted biological functions of significantly up-regulated and down-regulated circRNAs through pinpointing pathways relevant to their host genes. We indicated 10 signaling pathways that were possibly affected by HAE. These were Neurotrophin signaling pathway, Sphingolipid signaling pathway, Proteoglycans in cancer, Focal adhesion, cAMP signaling pathway, TNF signaling pathway, MAPK signaling pathway, Non − alcoholic fatty liver disease (NAFLD), and mTOR signaling pathway (Fig. 7A and 7B).

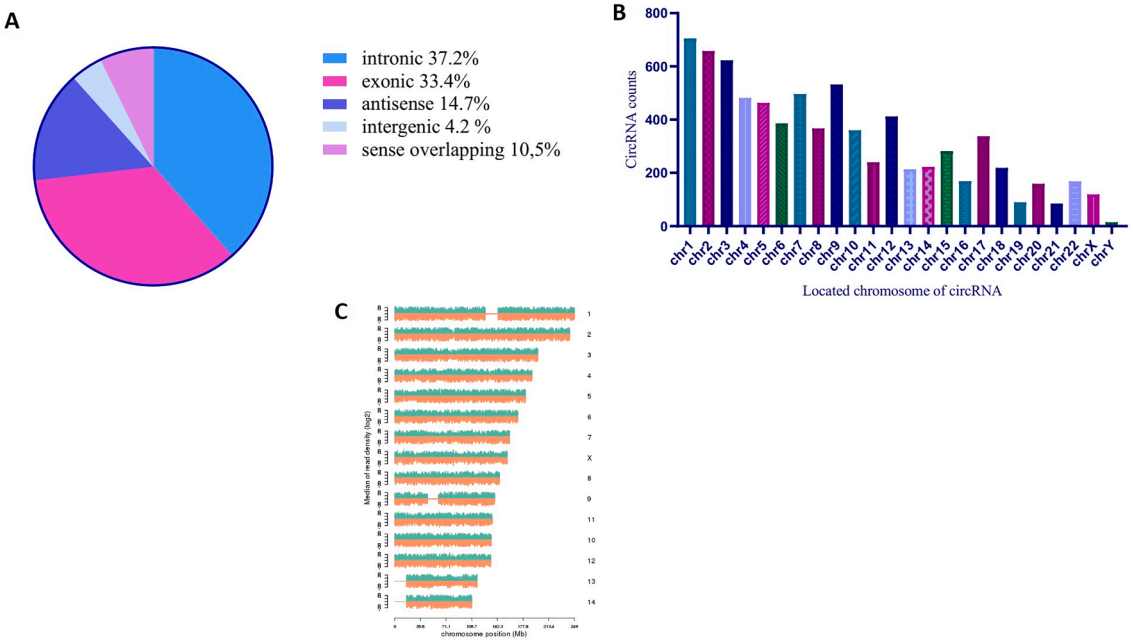

Fig. 2. Profiling of circular RNAs in human serum exosomes. A) Classification of identified circular RNAs based on the genomic origin. B) The distributions of identified circRNAs in different chromosomes. C) Distribution of reads in the chromosome.

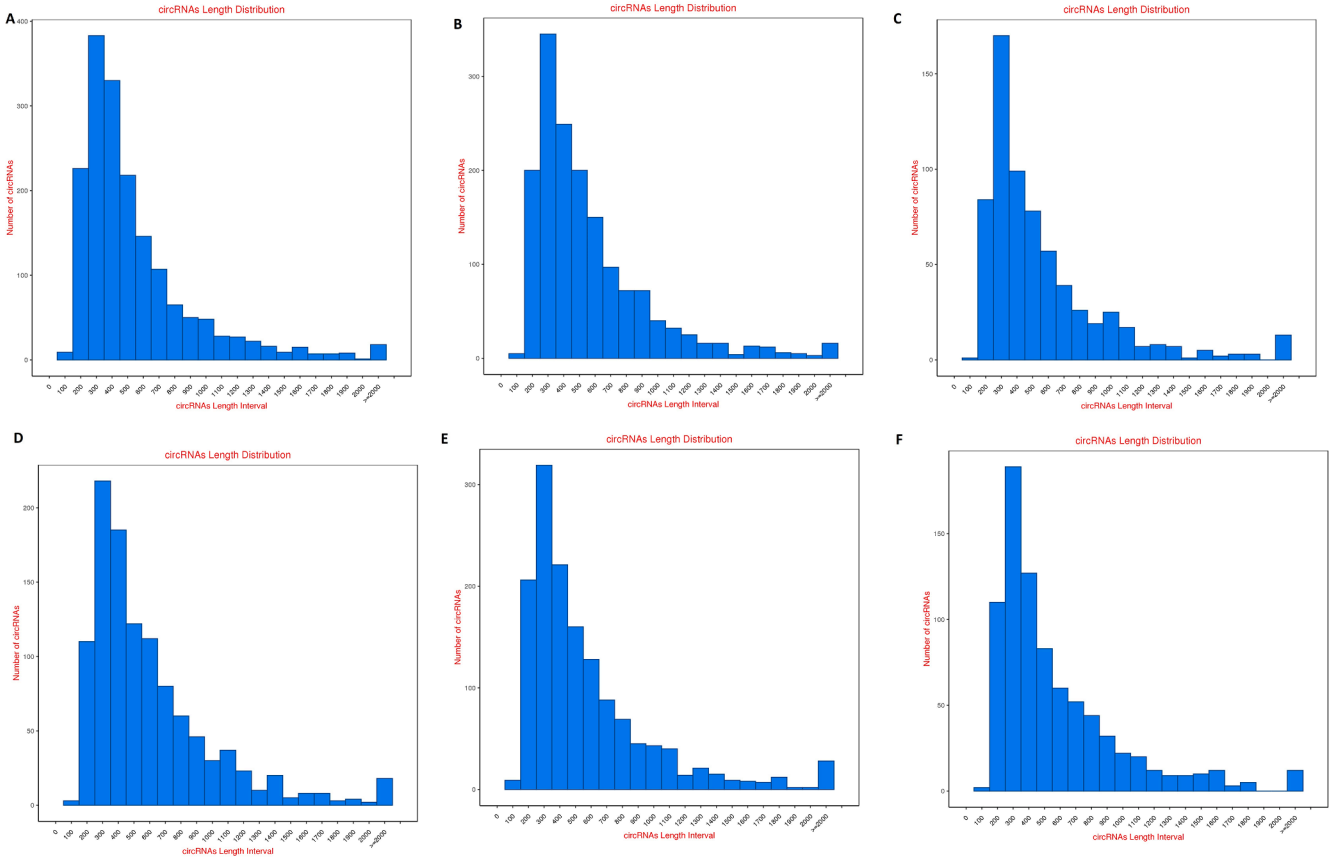

Fig. 3. Length distribution of the identified circRNAs. A) Control 1, B) Control 2, C) Control 3, D) Patient 1, E) Patient 2, F) Patient 3.

3.5. Network analyses

We constructed a circRNA-miRNA-target gene network for identified circRNAs using Cytoscape. The hsa-let-7a miRNA familiy potentially bind to identified circRNA and target genes were used to construct a network map (Fig. 8, Table S7).

4. Discussion

In here, we identified the exosomal circRNAs derived from serum in the HAE compared to the healthy control group according to the circRNA sequencing results, and then validated with RT-PCR. We found that significantly up-regulated circRNAs were hsa_circ_2204, hsa_circ_2290, hsa_circ_1854, hsa_circ_2436 and hsa_circ_2249; significantly downregulated circRNAs were hsa_circ_832, hsa_circ_160, hsa_circ_1332, hsa_circ_524, and hsa_circ_3988. Next, potential miRNA targets of circRNAs were predicted by sequence-matching-based miRNA target prediction software. In addition, hsa-let-7a miRNA familiy potentially bind to identified circRNA.

circRNAs have received significant attention as potential markers for some diseases. There are some reasons for this; stable structures (Jeck et al., 2013; Han et al., 2018), evolutionary conservation (Memczak et al., 2013; Zheng et al., 2016). abundant in several fluids, such as blood, saliva, serum, and even exosomes. These advantages make it possible to use circRNAs as liquid biopsy markers. (Li et al., 2015; Memczak et al., 2015; Dong et al., 2017). Previous research had reported that circRNAs have the function of promoting the growth and metastasis of cancer cells. For example, circular RNAs ciRS-7 and hsa_circ_0000069 have been found to be significantly overexpressed in colorectal cancer tissues and may serve as potential new and stable biomarkers for colorectal cancer diagnosis. It was screened the differentially expressed miRNAs by using a mircRNA microarray approach between infected liver tissues and normal tissues from HAE patients. It was observed that among the 2549 miRNAs detected, 3 (miR-1237-3p, miR-33b-3p and miR-483-3p) were significantly upregulated and 1 (miR-4306) was significantly upregulated (Ren et al., 2019). Although the roles of circRNAs in cancer and some diseases have been widely investigated, HAE-associated exosomal circRNA has not been investigated and characterized in the genome wide. We detected totaly 2203 circRNAs, 31 of them were significantly up-regulated and 28 of them were significantly down-regulated. Our results indicated that serum exosomal circ-RNAs have the potential to become a new biomarker for HAE diagnosis in the future because of its high sensitivity.

Bioinformatics analysis was carried out for the functions and mechanisms of circRNAs in HAE. We indicated some important functions or pathways that could explain possible mechanisms of increased circRNAs in HAE. For instance, up-regulated and down-regulated circRNAs are linked to Cancer progression and Proteoglycans in cancer. Detected circRNAs were related to focal adhesion that is an important mechanism and activated by multiple stimuli and functions as a biosensor or integrator to control cell motility (Mitra et al., 2005). Despite being known for over 60 years, the cAMP signal still reveals new functional details. The therapeutic intervention of its activities therefore requires further elucidation of its role in individual cell types and its links with other signaling and metabolic pathways (Raker et al., 2016). Interestingly, the circRNAs detected from this study were found to be related.

Two different tumor necrosis factors (TNFs), first isolated in 1984, were found to be cytotoxic to tumor cells and cause tumor regression in mice. Research over the past two decades has demonstrated the existence of a superfamily of TNF proteins consisting of 19 members that signal through 29 receptors. These ligands regulate normal functions such as immune responses, hematopoiesis and morphogenesis, as well as tumorigenesis, transplant rejection, septic shock, viral replication, bone

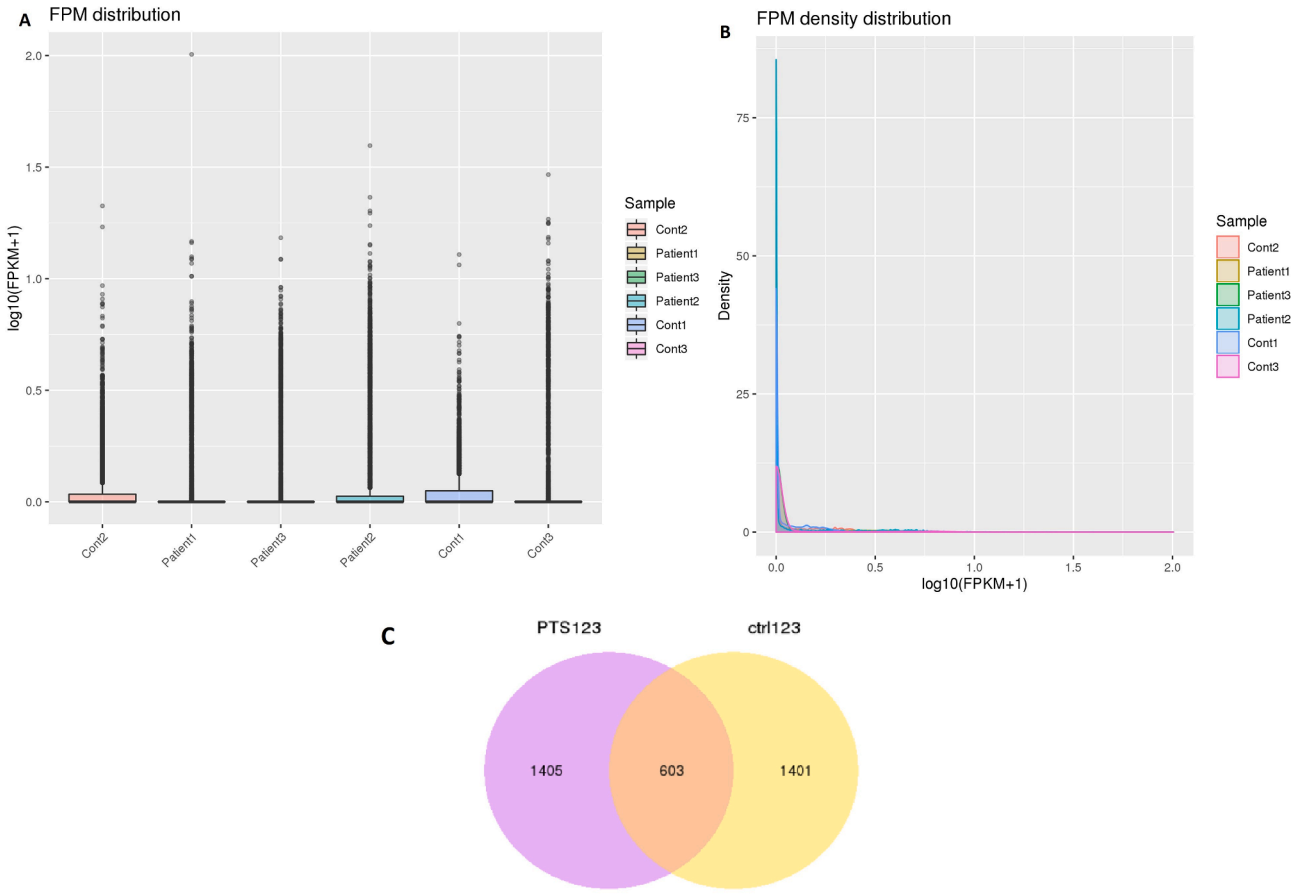

Fig. 4. FPM distribution diagram and Ven diagram for each group. A) FPM_violin. B) FPM_density. C) Venn diagram of differential expressed circRNAs. Each violin has five statistical magnitudes (max value, upper quartile, median, lower quartile and min value). The violin width shows the gene density. The x-axis is gene's log10 (FPM) value. The y-axis is the density of log10(FPM)value. The circles represents the total number of circRNAs in a single comparison group. The overlapped part is the number of circRNAs expressed in all comparison groups.

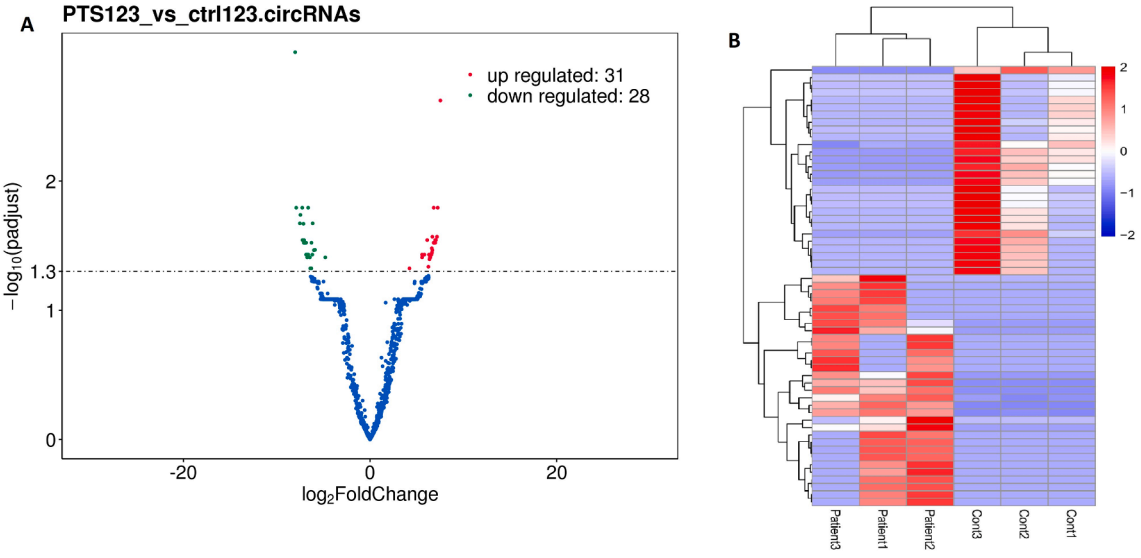

Fig. 5. Filtering of different expression of circRNAs and clustering of differential circRNAs. A) Volcano plot can be used to infer the overall distribution of different expression circRNAs. B) Heat Map analysis of circRNAs. The overall FPM cluster analysis result. clustered by log10(FPM + 1) value, red represents circRNAs with high expression level, and blue represents circRNAs with low expression level. The color from red to blue represents the log10(FPM + 1) value from large to small. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

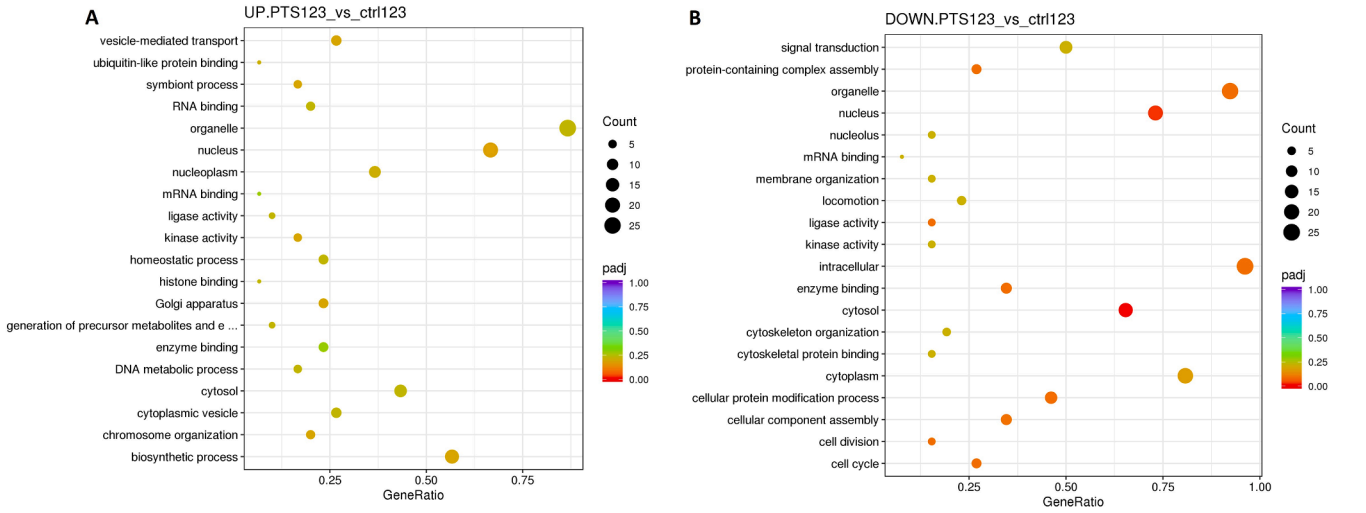

Fig. 6. Illustration of topGO DAG. A) Up-regulated circRNAs. B) Down-regulated circRNAs. Each node represents a GO term, and TOP10 GO terms are boxed. The darker the color is, the higher is the enrichment level of the term. The name and p-value of each term are present on the node.

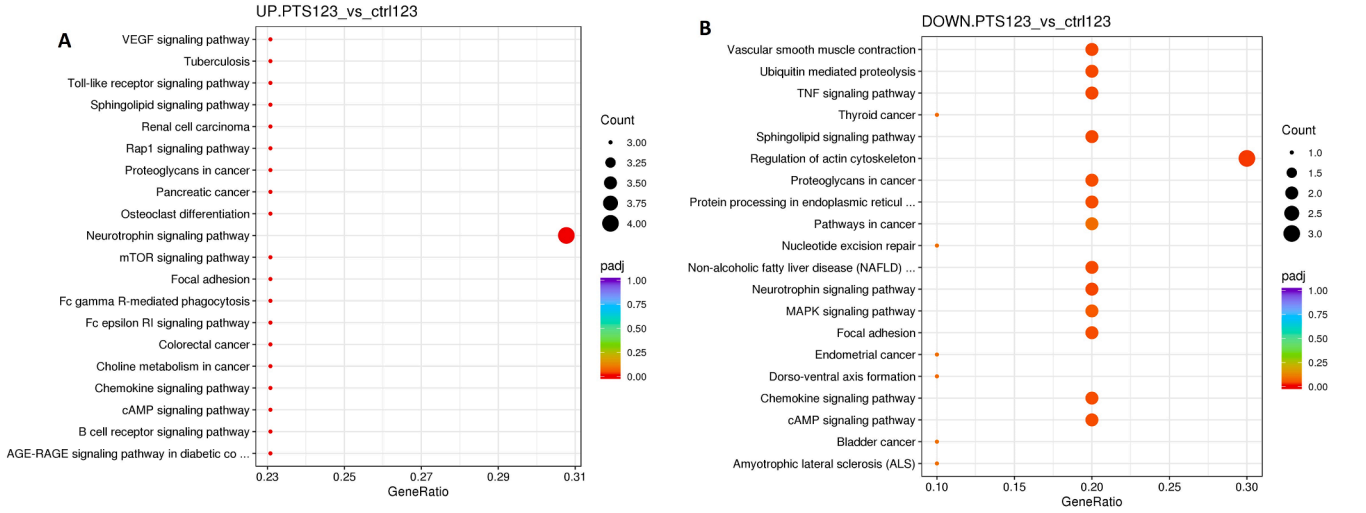

Fig. 7. Diagram Showing Significantly Enriched KEGG Pathway. A) Up-regulated circRNAs. B) Down-regulated circRNAs.

resorption, rheumatoid arthritis and diabetes; namely their role as 'double-edged swords'. These cytokines either induce cellular proliferation, survival, differentiation or apoptosis (Aggarwal, 2003). The circRNAs detected in this study were associated with the TNF alppha pathway, indicating that these circRNAs play an important role in the inflammatory process.

At least three families of MAPKs have been characterized: extracellular signal-regulated kinase (ERK), Jun kinase (JNK/SAPK), and p38 MAPK. The above effects are mediated by the regulation of cell cycle motor and other cell proliferation-related proteins (Zhang and Liu, 2002). The mechanistic Rapamycin Target (mTOR) coordinates eukaryotic cell growth and metabolism with environmental inputs, including nutrients and growth factors. Extensive research over the past two decades has established a central role for mTOR in regulating many essential cell processes, from protein synthesis to autophagy, and dysregulated mTOR signaling plays a role in the progression of cancer and diabetes as well as the aging process (Saxton and Sabatini, 2017). This study suggested that HAE related circRNAs could be play role in autophagy and apoptosis.

The most important limitation of this study is the low sample size. More detailed analysis of the identified circRNAs is required before inclusion in clinical practice. In addition, cohort analysis could not be performed due to the short time allocated for the project and the limitations in obtaining patient information. Despite these limitations, we think that the data obtained from the current project is a first for HAE disease. In addition, we hope that the identified candidate circRNAs will shed light on future large-scale projects in this field.

In conclusion, differentially expressed exosomal circRNAs derived-serum in HAE were found with high-throughput sequencing analysis and verified using RT-qPCR. Identified circRNAs, hsa_circ_0002204, hsa_circ_0002290,hsa_circ_0001854,hsa_circ_0002436,hsa_circ_0002249,hsa_circ_0001743,hsa_circ_0001699,hsa_circ_0002357, hsa_circ_0002206,hsa_circ_0001698,hsa_circ_000832,hsa_circ_000160, hsa_circ_0001332, hsa_circ_000524, hsa_circ_0003988, hsa_circ_000875, hsa_circ_0001444,hsa_circ_0004047, hsa_circ_0004150, hsa_circ_0004187 could be the potential exosomal circRNA based biomarkers related to HAE in human. Our study suggest that a theoretical basis for investigating the role of these differentially expressed circRNAs in HAE pathogenesis, but the mechanisms underlying circRNA in HAE development still need further investigation.

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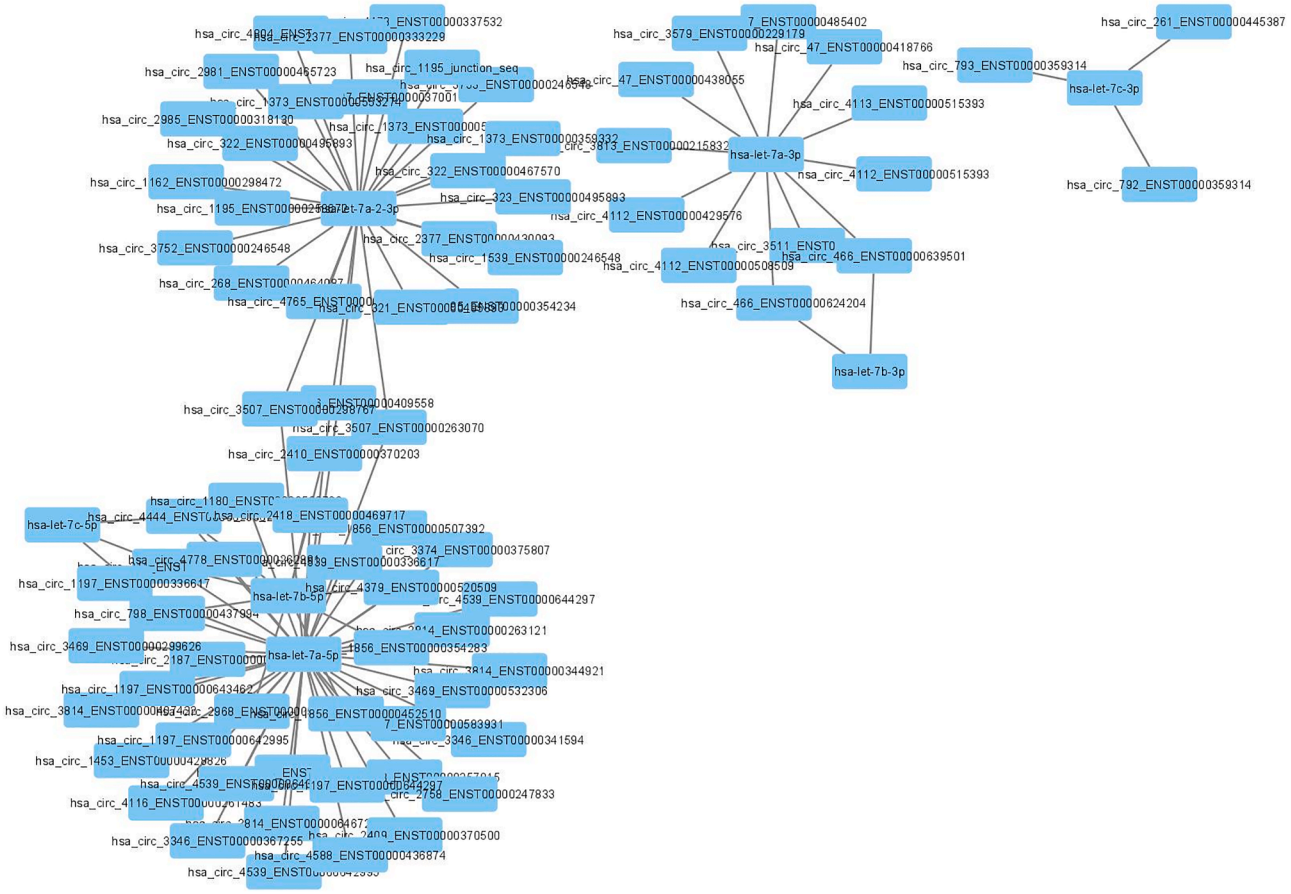

Fig. 8. CircRNA-miRNA-mRNA network prediction and analyses. The possible binding of miRNAs and mRNAs to circRNAs.

Number: 8751).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2021.146161.

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